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The post-thaw quality of ram sperm held for 0 to 48 h at 5 °C prior to cryopreservation☆

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Abstract

The effects of holding diluted ram semen at 5 °C for up to 48 h prior to cryopreservation were investigated. Semen from six rams was collected by electro-ejaculation in the autumn and again from six different rams in the spring. The sperm concentration and motility were determined using spectrophotometry and computerized automated semen analysis, respectively. Samples were diluted at 23 °C to 400×10^6 cells/ml in a one-step Tris–egg yolk–glycerol (5%, v/v) media, cooled to 5 °C over 2 h and maintained at 5 °C for the duration of the experiments. Aliquots were loaded into 0.5 ml French straws at 0, 24 or 48 h after cooling, frozen in liquid nitrogen vapor for 12–13 min, 4.5 cm above the liquid nitrogen, and plunged into liquid nitrogen for storage. After thawing, autumn samples frozen after 0, 24, or 48 h of storage exhibited similar percentages of motility (29, 31, 36%, respectively), progressively motility (16, 15, 17%, respectively), plasma membrane integrity (28, 35, 29%, respectively) and live acrosome-reacted cells (0.4, 0.6, 0.8%, respectively; $P > 0.05$). In addition, the quantity of sperm that bound to hen's egg perivitelline membranes after being held at 5 °C for 0, 24, or 48 h was not significantly different when the values were expressed as means of the quantity of sperm (155, 177, 106 sperm, respectively) or as the proportion of sperm inseminated (0.39, 0.49, 0.34, respectively; $P > 0.05$). Likewise, ram sperm collected in the spring and frozen at 0, 24 and 48 h after cooling had similar ($P > 0.05$) total motility (21, 25, 20%, respectively), progressive motility (14, 15, 11%, respectively), plasma membrane integrity (26, 33, 31%, respectively) and live acrosome-reacted cells (3.7, 3.5, 3.2%, respectively; $P > 0.05$). The 0 h holding time had significantly

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less sperm bound to a hen's egg perivitelline membrane compared to the 48 h holding time (250 and 470 sperm, respectively) although the 24 h holding time was not different from the 0 or 48 h holding time (281 sperm; $P < 0.05$) but analysis of the proportion of the total sperm inseminated resulted in no significant differences observed ($P > 0.05$). These results indicate that ram sperm can be held at 5 °C for up to 48 h prior to freezing with no injurious effects on motility, membrane integrity, or fertilizing potential as indicated by membrane binding ability.

Keywords: Spermatozoa; Cryopreservation; Holding time; Ram

1. Introduction

The goal of the National Animal Germplasm Program is to bank viable germplasm from agricultural species; however, most animals of interest are not within reasonable proximity of our facility. Consequently, it is necessary to collect and transport semen samples from the place of collection to our laboratory while maintaining the semen quality before and after cryopreservation. Ideally, a 48 h time span from collection to cryopreservation would be most opportunistic for our needs. These same barriers exist for sheep producers, as little infrastructure exists to efficiently collect, process, and cryopreserve ram semen for artificial insemination.

Semen from many species such as bulls (Graham et al., 1957; Batra et al., 1981; Foote and Kaproth, 2002), boars (Kikuchi et al., 1998; Guthrie and Welch, 2005), stallions (Crockett et al., 2001; Backman et al., 2004) and rams (Jones and Martin, 1964; Lightfoot and Salamon, 1969; Fiser and Batra, 1984) can be cooled to and maintained at 5 °C for time periods up to 24 h, depending on species, prior to cryopreservation and still have acceptable post-thaw sperm quality and fertility. Ram sperm held at 6 °C for 24 h, or up to 12 days maintain acceptable motility (53%, Tiwari and Sahni, 1976; and 50%, Lopez-Saez et al., 2000), but documentation concerning cryopreserving ram sperm following incubations of up to 48 h have not been reported. Therefore, the objectives of this study were to determine the effects of holding time prior to cryopreservation on post-thaw ram sperm quality and fertilizing potential.

2. Materials and methods

2.1. Semen collection, pre-freeze evaluation, and cryopreservation

All chemicals were reagent grade and were purchased from Sigma-Aldrich, St. Louis, MO (see footnote to the title, unless otherwise noted).

Semen was collected from rams in the autumn ($n = 6$) and in the spring ($n = 6$) using an electro-ejaculator as described by Evans and Maxwell (1987). The sperm concentration was determined using spectrophotometry (Hammerstedt, 1975) and the sperm motility estimated using phase contrast microscopy (400×). All samples had an initial total motility of at least 70%.

Egg yolk–Tris medium (300 mM Tris, 28 mM glucose, 95 mM citric acid, 2% (v/v) glycerol, 15% egg yolk, 1 mg/ml streptomycin sulfate and 0.06 mg/ml benzylpenicillin; Sanchez-Partida et al., 1998) was warmed to 37 °C and the samples diluted to 400×10^6 cells/ml in one step in a plastic 50 ml centrifuge tube.

Samples were cooled to 5 °C over 2 h by placing them in a beaker with 100 ml of 37 °C water and placing the beaker in a 5 °C room where they remained until freezing. After cooling to 5 °C, samples were split into three aliquots and one aliquot (time 0) was loaded into 0.5 ml French straws. The straws were frozen in liquid nitrogen vapor, 4.5 cm above liquid nitrogen, for 12 min and then the straws were plunged into liquid nitrogen for storage. The remaining aliquots were held at 5 °C for 24 or 48 h prior to being packaged and frozen, in the same manner.

2.2. Motility analysis

Cryopreserved samples were thawed for 30 s in a 37 °C water bath and the motility analyzed using computer automated semen analysis (CASA; Hamilton Thorne Motility Analyzer, Beverly, MA, see footnote to the title). An aliquot of each sample (50 μl) was diluted with 150 μl of Tris-buffered saline (Purdy and Graham, 2004) and 5 μl of this solution was placed on a Standard Count Analysis Chamber (Spectrum Technologies, Healdsburg, CA, see footnote to the title) and analyzed for motility. The CASA was setup with the following settings: 50 frames acquired, frame rate of 60 Hz, minimum contrast of 60, minimum cell size of 5 pixels, VAP cutoff of 20 μm, progressive minimum VAP cutoff of 50 μm/s, VSL cutoff of 30 μm/s, static head size of 0.24–3.66, magnification of 1.89 and a minimum of 1000 sperm were observed for motility analysis.

2.3. Acrosomal integrity and viability analysis

Aliquots of frozen-thawed samples (10×10^6 cells) were diluted to 0.5 ml in Tris-buffered saline (200 mM Tris, 65 mM citric acid, 55 mM glucose; Purdy and Graham, 2004) and treated with FITC-PNA (10 μl of a 1 mg/ml solution in water) and propidium iodide (PI; 5 μl of a 2.4 mM solution in water; Molecular Probes, Eugene, OR) to determine the acrosomal integrity (FITC-PNA probe) and the plasma membrane integrity (PI probe), respectively. The samples were stained at 23 °C for 10 min prior to analysis using a Coulter Electronics Epics V flow cytometer (Coulter Electronics Miami, FL). The flow cytometer was equipped with an argon laser tuned to 488 nm at 100 mW of power and the following filters: a 515 nm long pass filter, a 525 nm band pass filter to detect FITC-PNA fluorescence, and a 630 nm long pass filter to detect PI (Flesch et al., 1998). A minimum of 50,000 sperm were analyzed for each sample. Analysis of sperm in this manner provides populations of dead sperm that are non-acrosome-reacted (NAR), dead sperm that are acrosome-reacted (AR), live sperm NAR, and live sperm AR (Flesch et al., 1998).

2.4. Ability of ram sperm to bind to hen's egg perivitelline membranes

The binding ability of cryopreserved ram sperm was determined using a procedure from Cramer et al. (1994) and modified by Moce-Cervera and Graham (personal communication).

Chicken egg yolks were separated from the whites and the perivitelline membrane was opened to release the contents. The membrane was washed in Dulbecco's phosphate buffered saline (171 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) until yolk-free and the membrane was spread out in a petri dish. The membrane was then cut into uniform pieces using a cork borer (1 cm diameter) and placed into 1.5 ml centrifuge tubes containing 0.5 ml of TALP (Graham et al., 1986). Sperm samples were thawed as described previously, and 30,000 motile cells, determined from the motility analyses, were mixed with each membrane and incubated at 39 °C. After 1 h of incubation the samples were stained with 5 μl of SYBR-14 (20 μM in Me_2SO; Molecular Probes, Eugene, OR) and incubated for an additional hour. The membrane samples were washed three times with DPBS to remove unbound cells, placed on a microscope slide, and covered with a coverslip. The number of sperm bound to each membrane in 10 fields was counted at 400× magnification on a Nikon Eclipse E600 fluorescent microscope equipped with a 531 nm bandpass filter set to observe the sperm labeled with SYBR-14. Three replicated analyses were performed for each cryopreservation time per ram using membrane explants from three eggs.

3. Statistics

Differences were determined using the General Linear Model with ram, holding time, and ram by holding time interaction as the main effects with separate analyses performed for each season (SAS Institute Inc., 1985). The means were separated using the lsmeans diffs procedure (SAS Institute Inc., 1985). Differences in the mean number of frozen-thawed ram sperm that bound to membranes by holding time was determined using ANOVA and the means were separated using Duncan's multiple range test (SAS Institute Inc., 1985). In addition, differences in ram sperm that bound, as a proportion of the number of sperm inseminated, were determined using ANOVA and the means were separated using Duncan's multiple range test (SAS Institute Inc., 1985). The correlation between total sperm added to membranes and the number of sperm binding was evaluated using the CORR procedure of SAS (SAS Institute Inc., 1985).

4. Results

No holding time treatment differences in the proportion of post-thaw motility, progressive motility, plasma membrane integrity or live acrosome-reacted ram sperm were observed in either season ($P > 0.05$; Table 1). In addition, no differences were detected in the mean number of sperm that bound to hen's egg perivitelline membranes by holding time in autumn, but holding time 0 in spring had significantly less sperm bound to the membranes compared to holding time 48 h, although holding time 24 was not different from holding time 0 or 48 h ($P < 0.05$). When analysis of sperm binding as a proportion of the number of sperm inseminated was performed, no differences were detected within either season ($P > 0.05$).

Differences by ram in the number of sperm that bound to hen's egg perivitelline membranes were observed within a holding time and season but this varied by the

Table 1
The post-thaw motility, membrane integrity, and membrane binding assay analysis results for ram sperm held for 0, 24, or 48 h at 5 °C prior to cryopreservation, by season ($n = 6$ rams per season)

Season	Sperm parameter	Holding time (h)			S.E.M.[1]
		0	24	48	
Autumn	Motility (%)	29	31	36	7.7
	Progressive motility (%)	16	15	17	4.1
	Plasma membrane integrity (%)	28	35	29	6.7
	Live acrosome-reacted (%)	0.4	0.6	0.8	0.3
	Membrane binding (number of sperm)	155	177	106	29
	Membrane binding (% of sperm bound)	0.39	0.49	0.34	0.04
Spring	Motility (%)	21	25	20	5
	Progressive Motility (%)	14	15	11	3.1
	Plasma membrane integrity (%)	26	33	31	4.1
	Live acrosome-reacted (%)	3.7	3.5	3.2	1.2
	Membrane binding (number of sperm)	250^{a}	281^{ab}	470^{b}	112
	Membrane binding (% of sperm bound)	0.43	0.58	0.72	0.14

[ab] Superscripts indicate significant differences within that row ($P < 0.05$).
[1] The standard error of the mean (S.E.M.) is for the data in the corresponding row.

type of analysis (mean number of sperm bound or proportion of inseminated sperm bound) performed (Table 2). No significant correlation between the total number of sperm used to inseminate the membranes and the number of sperm bound was observed ($P > 0.05$).

Table 2
The mean numbers and the proportion of frozen-thawed ram sperm that bound to chicken oocyte perivitelline membranes the ram sperm had been frozen at time 0 (immediately after cooling to 5 °C) or after holding at 5 °C for 24 or 48 h

Season	Ram	Holding time (h)[1]		
		0	24	48
Autumn	1	105/0.14	186^{abc}/0.27	112^{b}/0.19
	2	91/0.19	99^{bc}/0.19	40^{b}/0.07
	3	191/0.01	145^{abc}/0.06	71^{b}/0.03
	4	183/0.07	324^{a}/0.09	270^{a}/0.2
	5	154/0.1	53^{c}/0.04	65^{b}/0.08
	6	204/0.28	258^{ab}/0.33	80^{b}/0.1
	S.E.M.[2]	82/0.08	57/0.06	50/0.05
Spring	7	616^{a}/0.08	565^{a}/0.13^{a}	1122^{a}/0.26
	8	142^{b}/0.13	179^{c}/0.17^{a}	459^{b}/0.28
	9	273^{b}/0.4	241^{bc}/0.24^{ab}	243^{b}/0.25
	10	218^{b}/0.12	439^{ab}/0.4^{b}	492^{b}/0.31
	11	174^{b}/0.08	148^{c}/0.07^{a}	287^{b}/0.1
	12	79^{b}/0.05	114^{c}/0.15^{a}	220^{b}/0.25
	S.E.M.[2]	103/0.17	67/0.06	121/0.08

[abc] Different superscript letters indicate differences by column and analysis within a season ($P < 0.05$).
[1] The value in each cell is the mean number of sperm from three replicates and the proportion (%) of inseminated sperm that bound.
[2] Standard error of the mean (S.E.M.).

5. Discussion

Holding spermatozoa at temperatures below body temperature prior to insemination, cryopreservation, or both, is commonly done in many species with acceptable results. Holding bull sperm for up to 18 h at 5 °C prior to cryopreservation does not result in a decline in proportions of motile sperm post-thaw (Foote and Kaproth, 2002), nor does it alter non-return rates (Graham et al., 1957; Foote and Kaproth, 2002). Stallion sperm may also be held at low temperatures (5 or 15 °C) for 18 h prior to cryopreservation, but the total and progressive motility are significantly lower after thawing compared to samples frozen immediately after cooling to 5 °C (Backman et al., 2004). The pregnancy rates obtained using stallion sperm held for 18 h, or frozen immediately after processing were not significantly different (53% versus 70%, respectively; $P > 0.05$) but the lack of a statistical difference may be an artifact of the sample size used in this study ($n = 30$ mares per treatment group; Backman et al., 2004). In addition, boar sperm are commonly held for extended periods of time (up to 24 h) at reduced temperatures (15 °C) prior to cryopreservation, and no differences with in vitro oocyte penetration are observed (Eriksson et al., 2001). Pregnancy rates determined after insemination with frozen-thawed boar sperm held for either 3 or 24 h at 15 °C declined over time and the longer holding time resulted in a decrease in embryo number from 15 to 9 per litter (Guthrie and Welch, 2005).

Previous research investigating holding ram sperm at low temperatures reports variable results. Patt and Nath (1969) observed that ram sperm could be held at 5 °C prior to cryopreservation for 6 h in glycerolated media without an effect on the motility (51%) compared to samples that were exposed to glycerol at 5 °C for 1 h (51%), but both times were significantly different from samples held for 12 h at the same temperature (40%; $P < 0.05$). Shorter incubation times (0.75–6 h) at 5 °C have resulted in what is reported to be the optimal post-thaw motility and/or non-return rates (Jones and Martin, 1964; Lightfoot and Salamon, 1969; Watson and Martin, 1975; Fiser and Batra, 1984). Incubation of ram sperm for longer times (18 h) at 5 °C has resulted in declines in progressive motility (5% decrease) and kinetic ratings (0.5 point decrease; Fiser and Batra, 1984). It was hypothesized that incubating ram sperm in the manner presented here at 5 °C for times greater than 12–24 h in the presence of glycerol, may have a detrimental effect and result in a decline in the overall quality of the sperm, based on the literature previously presented. It was surprising to observe that holding ram sperm in the manner described here resulted in no significant differences in motilities, plasma membrane integrity, or acrosomal membrane integrity of live sperm at the three holding times tested. This observation is stated cautiously though, because logically the quality of sperm declines over time since sperm are terminal cells that contain no means of self-reparation. Furthermore, the analyses performed, motilities and membrane integrities, are very limited in the information that they provide, such that no one test will fully address the overall quality of a sperm sample.

The in vitro binding assay was first described by Cramer et al. (1994) for fertility assessment and later validated with in vivo assessment (Barbato et al., 1994). The assay was used with rooster sperm for in vitro assessment of fertility by Phillips et al. (1996) and Barbato et al. (1998) and also with turkeys for determining sub-fertile toms (Gill et al., 1999). This methodology was also examined by Barbato et al. (1998) using mouse, bull, horse, rooster, and human sperm and it was determined that the binding efficiency varied between species,

but the assay was sufficiently sensitive to detect differences in binding based on insemination dose. Thus, the insemination dose is important because the membrane can be saturated with sperm due to a limited number of binding sites available on a membrane (Gill et al., 1999). The number of motile ram sperm inseminated in these experiments (30,000) was selected based on preliminary experiments (data not reported) where a reasonable number of bound sperm could be counted in 10 microscope fields at 400× magnification. The range of binding, 40–1122 average sperm per membrane, demonstrates that saturation of the membrane with bound sperm was not achieved.

The results from the membrane binding assay are interesting because no differences were observed across holding times in the autumn, but the 48 h holding time in spring had significantly more sperm bound than the time 0 h spring holding time. When the sperm membrane binding was analyzed using number of sperm bound as a proportion of the total number of sperm inseminated, the results were very different. In this instance, differences in binding by ram were only detected in the spring at the 24 h holding time and no differences in holding time in either season were detected. Perhaps normalization of the data in this manner is a better method of analysis, even though no significant correlation of number of sperm inseminated with number of sperm binding was detected, because then this is a true comparison of proportions. Potentially, the reason for the differences in membrane binding is due to the different composition of ram seminal plasma and the changes in lipid content of ram sperm in the spring. Decreases in phospholipid content of ram sperm occur as the photoperiod increases (Evans and Setchell, 1979) consequently the sperm would potentially be more fusogenic which could account for the increase in the percentage of live acrosome-reacted sperm and the differences in membrane binding in the spring. The purpose for using equal numbers of motile sperm in the membrane binding assays was to 'balance' the inseminations, but obviously there were still differences beyond these observed characteristics of the sperm. Insemination doses based on plasma membrane or acrosomal membrane integrity may have produced different results but all of these sperm attributes have similar poor correlations to fertility when used by themselves (Graham, 2001). Therefore, other factors, or most probably, a combination of the sperm attributes, are obviously involved and these determine the degree of binding for a particular sample.

It is tempting to use the membrane binding assay to determine the potential fertility of a ram or a sperm sample because differences in rams can be observed (Table 2) and because others have used this methodology for this purpose (Gill et al., 1999). Instead, this assay is more properly used to evaluate the potential for a sperm sample to undergo capacitation and the acrosome reaction (Yanagamachi, 1984). To successfully fertilize an egg, or bind to a membrane, a sperm must complete a series of processes. In this particular instance the processes of capacitation and the acrosome reaction are being evaluated, and if the sperm are unable to do so, membrane binding will not occur (Barbato et al., 1998). The interpretation of results from this type of test is limited. Many factors such as retention of sperm adhesins, differences in ejaculates, and agglutination of sperm during incubation can distort the results (Waberski et al., 2005). Still, as membrane binding is required of a sperm, there is value to this assay and if further explored could potentially be used for improving reproductive performance from a particular male by adjusting the insemination dose to maximize binding/fertilization (Barbato et al., 1998).

Reports of seasonal differences in post-thaw quality and fertility of ram sperm are variable. Suffice it to say that results that demonstrate a deleterious effect of season on post-thaw quality and/or fertility during the increasing photoperiod (Colas and Brice, 1976; Fiser and Fairfull, 1983, 1986; Guerin et al., 1992; D'Alessandro and Martemucci, 2003) can be balanced by reports demonstrating that there is no effect of season on ram sperm quality and freezability (Maxwell, 1980; Curnock et al., 1984). Further confounding information demonstrates that seasonal differences may be attributed to breed, ram, and ejaculate (Salamon and Maxwell, 1995).

Comparisons of season were not performed in this research because different groups of rams were used in the different seasons and analyses were performed at two different times of the year. Therefore direct comparison would not have been appropriate. To be cautious, it should also be suggested that the membrane binding assay only approximates binding that would occur in vivo. Perhaps the increased binding that was observed in spring was due to a poor quality of the sperm due to the longer holding time and seasonal changes in the ram sperm membranes. These factors coupled with the potential cryo-capacitation that may have occurred, could potentially make the sperm that much more fusogenic and consequently more binding was observed when compared with the other holding times.

These results demonstrate that it is feasible to collect ram sperm and hold them for up to 48 h prior to cryopreservation without deleterious effects on the post-thaw sperm quality. This same technique can be used by producers to have semen samples frozen at commercial stud services that may not be in close proximity to their farm or ranch. Future work with ram sperm needs to be conducted to determine if ram sperm held for 24 or 48 h prior to freezing maintain their fertilizing potential and to determine the true value of the membrane binding assay.

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